

# EXPERIMENTAL MODEL OF OSTEOPOROSIS: COMPARISON BETWEEN OVARIECTOMY AND BOTULINUM TOXIN A

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## ABSTRACT

**Objective:** To evaluate whether Botulinum toxin-A (BTX-A) has a similar effect to that of ovariectomy (OVX) on bone regarding bone mineral densitometry. **Methods:** A total of 51 female rats were randomly divided into three groups of 17 animals each. The rats in the first group formed the control group, without any surgical procedure (Group 1). Group 2 received BTX-A while Group 3 was subjected to OVX. A total of 8 IU of BTX-A was injected into the right femoral region of all rats in Group 2. At baseline and 14 weeks later, bone mineral densities (BMD) of the left and right femurs of all rats in both groups were measured. **Results:** There was no statistically significant difference

between the groups with respect to baseline BMD. At the 14<sup>th</sup> week the BMD of the right femurs were statistically significantly higher in Group 1 than other groups, although there was no statistically significant difference between Groups 2 and 3. The mean BMD results of the left femur in Group 3 were statistically significantly lower than the results in Groups 1 and 2 at the 14<sup>th</sup> week. **Conclusion:** The results of the current study showed that BTX-A had a similar effect to that of OVX on osteoporosis regarding BMD. **Evidence Level I, Experimental, Controlled, Animal Study.**

**Keywords:** Botulinum toxins, type A. Ovariectomy. Osteoporosis.

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## INTRODUCTION

Osteoporosis is a chronic disease that affects millions people characterized by a decrease in bone mass and deterioration in bone micro architecture which leads to an enhance fragility of the skeleton, and therefore to a greater risk of fracture. Decreased physical activity and skeletal unloading may contribute to osteoporotic bone loss. Prolonged immobilization consequent to long periods of bed rest, decreased muscle function caused by neurological conditions and injuries, as well as weightlessness of spaceflight result in a very fast and severe bone loss attended by uncoupling between bone formation and resorption. Removal of physical loading decreases osteoblast function, whereas mechanical stimulation in general has been shown to have an anabolic effect on bone cell dynamics, bone mass, and quality.<sup>1</sup> Animal models provide more uniform experimental material and allow for extensive testing of potential therapies. The high cost and long time frame of clinical testing are other reasons why animal models play a crucial role in osteoporosis research.<sup>2</sup> Ovariectomy, change of diet, drugs, immobilization, breeding, central control of bone mass are commonly used for an osteoporosis animal model.<sup>3</sup> The ovariectomized (OVX) rat model

is most commonly used in research on postmenopausal osteoporosis. After ovariectomy, bone resorption exceeds bone formation initially, causing bone loss. Soon thereafter, bone remodelling reaches a steady state, where resorption and formation are balanced.<sup>4</sup>

Botulinum toxin type A (BTX-A) is one of seven serologically distinct neuromuscular blocking agents produced by the bacterium *Clostridium botulinum*.<sup>5</sup> BTX-A is highly specific for motor nerve terminals and has a high capacity for diffusion through muscle upon injection. These factors make BTX-A an ideal agent for induction of muscle weakness and paralysis. Our hypothesized was that immobilization of the extremity after BTX-A injection may causes regional osteoporosis in the injected side. The main purpose of this study was to evaluate whether BTX-A has a similar effect with OVX on bones regarding to bone mineral densitometry (BMD).

## MATERIALS AND METHODS

### Animal Model and Study Design

This experimental study was carried out in the Experimental Medicine and Clinical Research Unit (EMCRU) of Kocaeli University

All the authors declare that there is no potential conflict of interest referring to this article.

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after the approval of the Animal Research Ethical Committee was obtained. In the present study, 51 female Wistar Albino rats aged 3-3.5 months, weighing between 200-300g were used as experimental animals. The Animals were divided randomly in to three groups of seventeen animals each. The rats in the first group were control with no additional surgical procedure (Group 1). Group 2 received BTX-A while Group 3 was subjected to OVX. Total of 8 IU of BTX-A (Botox<sup>®</sup>, Allergan, Irvine, California) was injected into the right femoral region of all rats in group 2; 4 IU into the anterior muscle group and 4 IU into the posterior muscle group.<sup>6</sup> At the beginning and 14 weeks later, bone mineral density (BMD) of the left and right femurs of all rats in three groups were measured *in vivo* under ketamin anesthesia with a dual-energy X-ray absorbsiometry. After initial BMD measurements bilateral ovariectomy was performed to the rats in group 3. The rats were anaesthetized with an intra-peritoneal injection of ketamine (50 mg/kg-Ketalar<sup>®</sup>). 500 mg/kg ampicillin-sulbactam (Ampisid<sup>®</sup>) was injected intramuscularly to the rats. Rats were shaved and aseptically prepared and were operated by the same surgeon. OVX were performed by transverse ventral incision. All Rats were acclimatized to caged laboratory conditions and were allowed to feed with a standard diet and water ad libitum. The room temperature and humidity were maintained at 20–24°C and at 50–60%, respectively. The light cycle was fixed at 12 hours. Two animals in group 1, one in group 2 and two in group 3 died during the follow-up period (one during surgery) so 46 rats with 92 femur were evaluated finally. All animals in this study were sacrificed at 14<sup>th</sup> week by using high dose of ketamine.

### Bone densitometry

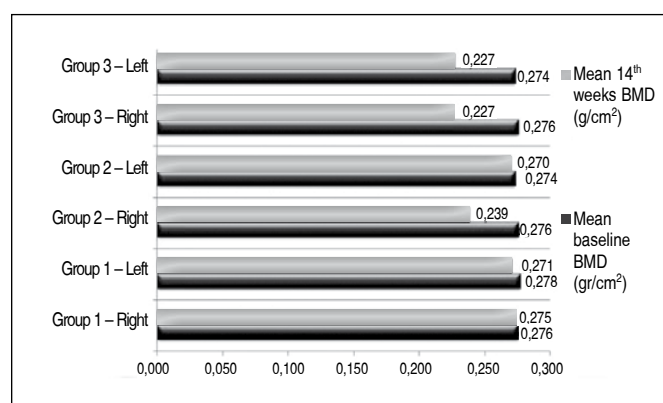
At the baseline and 14 week later, bone mineral density (BMD) of the femurs were measured *in vivo* under anesthesia with a dual-energy X-ray absorptiometer (Norland XR-36) using the small-animal software in each rat. Initially 51 rats with 102 femurs were evaluated while 46 rats with 92 femurs were measured at the end of 14 weeks. In all measurements the animals were placed in a supine position with a complete abduction of the hind limbs and each analysis was performed by the same researcher. The instrument was calibrated daily. The scan images were analyzed and BMD (in grams per square centimeter) (gr/cm<sup>2</sup>) of the metaphyseal zone of proximal femurs were determined.

### Statistical analysis

Each mouse had its BMD evaluated separately in twelve subgroups for the right and left femurs regarding to mean baseline and final (14<sup>th</sup> week). Mean and standard deviation (SD) were calculated for descriptive statistics of continuous variables and median values for discrete variables. Kolmogorov-Smirnov test was used to analyse the normality of data. The means of groups were analyzed by using ANOVA. *Post hoc* Bonferroni's multiple comparison procedure was used to determine which values were significantly different. Paired t-tests were used to compare means for BMD data between the subgroups. Two tailed hypothesis was considered in the analyses and a significant difference was accepted while  $p \leq 0.05$ . SPSS 15.0 Software for Windows (SPSS Inc., Chicago, IL, USA) was used in the evaluation of statistical analysis.

## RESULTS

The mean BMDs at the beginning and final follow-up of right and left femurs of all animals as show in Figure 1. There was no statistically significant difference between the groups with respect to baseline BMD of right and left femurs ( $p = 1,000$  and  $p = 0,788$  respectively). With respect to 14<sup>th</sup> week BMD of the right femurs, there was statistically differences between the groups ( $p=0,000$ ). Bonferroni test showed the difference was sourced from group 1 when compared with the others while there was no statistically significant difference between groups 2 and 3 ( $p = 0,256$ ). The mean BMD results of the left femurs in group 3 were statistically lower than the results in groups 1 and 2 at the time of 14<sup>th</sup> week. The statistically significance results of all subgroups were given in Table 1.



**Figure 1.** Mean baseline and 14<sup>th</sup> weeks bone mineral densitometry values of rats in control, Botulinum Toxin-A injected and ovariectomized groups.

## DISCUSSION

BMD' decrease after ovariectomy is expected in both femurs.<sup>4</sup> Also BTX-A injection decreased BMD of the right femurs of the rats as same as OVX while there was no statistically significant difference between the left femurs of same animals in group 2 and the rats in control group.

Several experimental interventions are used to induce osteopenia and osteoporosis in the rats.<sup>4</sup> The rate of bone loss in male and female rats is highly dependent upon the method used to induce osteoporosis, and the site evaluated and if that loss refers to the cancellous or cortical bone.<sup>4,7</sup> All experimental osteoporosis protocols can be implemented in skeletally immature or mature rats.<sup>8</sup> Although rats reach sexual maturity at the age of 2.5 months, their skeleton is considered mature after the age of 10 months.<sup>7</sup> If skeletally immature rats are involved, then a low peak bone mass is achieved, a fact that is considered to be a high risk factor for human osteoporotic fractures. This trait is why the skeletally immature rat is an appropriate animal model in the research of endocrine, nutritional and environmental factors, all of which can influence peak bone mass.

The skeletally mature rat is an appropriate animal model for the research of postmenopausal and immobilization osteoporosis.<sup>9</sup> After OVX, bone resorption exceeds bone formation initially, causing bone loss. Soon thereafter, bone remodelling reaches a steady state, where resorption and formation are balanced.<sup>4</sup>

**Table 1.** Statistical differences between subgroups obtained by Paired t-tests.

	Mean Baseline BMD-Group 1R (N=17)	Mean Baseline BMD-Group 1L (N=17)	Mean Baseline BMD-Group 2R (N=17)	Mean Baseline BMD-Group 2L (N=17)	Mean Baseline BMD-Group 3R (N=17)	Mean Baseline BMD-Group 3L (N=17)	Mean 14th weeks BMD-Group 1R (N=15)	Mean 14th weeks BMD-Group 1L (N=15)	Mean 14th weeks BMD-Group 2R (N=16)	Mean 14th weeks BMD-Group 2L (N=16)	Mean 14th weeks BMD-Group 3R (N=15)	Mean 14th weeks BMD-Group 3L (N=15)
Mean Baseline BMD-Group 1R (N=17)	1,000											
Mean Baseline BMD-Group 1L (N=17)	0,710	1,000										
Mean Baseline BMD-Group 2R (N=17)	1,000	0,750	1,000									
Mean Baseline BMD-Group 2L (N=17)	0,858	0,608	0,841	1,000								
Mean Baseline BMD-Group 3R (N=17)	1,000	0,829	1,000	0,841	1,000							
Mean Baseline BMD-Group 3L (N=17)	0,766	0,577	0,777	0,939	0,715	1,000						
Mean 14th weeks BMD-Group 1R (N=15)	0,840	0,678	0,885	0,937	0,955	0,739	1,000					
Mean 14th weeks BMD-Group 1L (N=15)	0,728	0,301	0,657	0,700	0,594	0,743	0,474	1,000				
Mean 14th weeks BMD-Group 2R (N=16)	0,000*	0,000*	0,000*	0,001*	0,003*	0,000*	0,000*	0,000*	1,000			
Mean 14th weeks BMD-Group 2L (N=16)	0,485	0,412	0,429	0,069	0,476	0,606	0,364	0,865	0,001*	1,000		
Mean 14th weeks BMD-Group 3R (N=15)	0,000*	0,000*	0,000*	0,000*	0,000*	0,000*	0,000*	0,000*	0,256	0,000*	1,000	
Mean 14th weeks BMD-Group 3L (N=15)	0,000*	0,000*	0,000*	0,000*	0,000*	0,000*	0,001*	0,000*	0,350	0,000*	0,727	1,000

\* indicates a significant difference while  $p \leq 0.05$ . Dark gray painted areas show statistically insignificant differences between BTX-A injected side and OVX groups. N, indicates numbers of evaluated femurs. R= Right, L= Left, BMD= Bone mineral density.

In cortical bone, enlargement of the marrow cavity is an indirect measure of bone loss. This enlargement in the diaphysis of long bones is due to increased endosteal bone resorption<sup>10</sup> and periosteal bone apposition.<sup>11</sup> Endosteal resorption and the simultaneous periosteal bone formation result in a very slow rate of cortical bone loss.<sup>11</sup> The main concerns about OVX are its irreversible and generalize effects on bones and other systemic. But BTX-A is easy to apply and has reversible and regional osteoporotic effect on bones.

Another method for inducing osteoporosis in rats is through immobilization. There are several methods of immobilization.<sup>4,12,13</sup> Because of the regional acceleratory phenomenon, the rate of bone loss is more fast after surgical methods than immobilization.<sup>14</sup> In the immobilization model, the bulk of bone loss occurs in the hind limbs, because they are the sites of greatest mechanical loading, but in general, the rate of bone loss is faster in cancellous than in cortical bone. This difference can partly be attributed to the surface to volume ratio, which is increased in cancellous bone.<sup>7</sup> In the current study BTX-A was used as a method of immobilization and had similar result on BMD with OVX. Nevertheless; the lack of fast mobilization due to paralyze of right femurs of the rats in group 2, there was

slight and statistically insignificant decrease in the left femurs with respect to BMD.

Recently Aydin *et al.*<sup>15</sup> reported their results regarding the effect of BTX-A on fracture healing. They reported statistically significant increase in union, spongy bone formation and bone marrow organization scores as well as elastic modulus value of BTX-A injected sides. They concluded that BTX-A administration increases the healing power in a relatively fixated fracture and decreases the callus diameter as if rigid fixation had been performed.<sup>15</sup> Nevertheless, Hao *et al.*<sup>16</sup> aimed to investigate whether local muscle atrophy and dysfunction affect fracture healing in a rat femur fracture model by using Botulinum toxin A (BTX-A). They concluded that; biomechanical testing indicated that the femurs of the BXTA-treated side exhibited inferior mechanical properties compared with the control side. The inferior outcome following BXTA injection, compared with saline injection, in terms of callus resistance may be the consequence of unexpected load and mechanical unsteadiness caused by muscle atrophy and dysfunction. Based on this information; it may be mentioned that the usage of BTX-A instead of OVX is not recommended in the study which evaluate fracture healing and osteoporosis simultaneously.

The association of both methods, OVX and immobilization can combining the advantages of both and also can markedly reduce the time when bone mass loss becomes apparent, especially for cortical bone.<sup>17,18</sup> The current study mainly suffers from the lack of data on the effect of OVX and immobilization in same subjects. Although biomechanical characteristics, cortical and cancellous bone thickness and also muscle weights to investigate atrophy were not evaluated, the current study showed BTX-A had similar

effect on BMD with OVX which may be use further investigations about experimental animal osteoporosis models.

## CONCLUSION

The results of the current study showed that BTX-A has a similar osteoporotic effect with OVX on bones regarding bone mineral densitometry in rats that has been common used for animal model of osteoporosis.

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